AMENDMENT TO THE TITLE

Please amend the title accordingly:

-- Diagnostic Kit for Detecting Pulmonary and Extra Pulmonary Tuberculosis --

AMENDMENTS TO THE SPECIFICATION

Please replace the paragraph beginning on page 1, line 5 and ending at line 16, with the following rewritten paragraph:

-- The conventional methods for detecting tuberculosis is time consuming & labour-intensive. Acid-fast bacilli (AFB) ~~consuming AFB~~ staining is considered to be insensitive (requiring 10,000 organism/ml of sputum for smear positive result with 100.times. microscope, refer Todar's Text Book of Bacteriology Online). ELISA-KP 90 is also known to be of low sensitivity and specificity ~~and specificity~~ (cut-off value >1.0 +ve, and <0.8 -ve test result) and requires sophisticated infrastructure as also the hypersensitivity based Tuberculin Skin Test (Montaux test), which lacks sensitivity, and specificity In BOG vaccinated patient ~~(Constantin P. et. al., Inf & Imm 1998; 66)~~. In the same way MYCODOT is inconvenient for HIV correlated individuals (14). ~~(refer G. R. Somi et. al., Int J Tubercle and Lung Disease, 19999, vol 3)~~ ~~and~~ Bactec-460 radiometric system (Becton Dickinson Instrument Systems, Sparks, MD. USA) is sensitive and is being used globally, but it took 5-10 days time for interpretation of the results and need for safe disposal of the radioactive waste products ~~and~~ whereas the Roche molecular system PCR based product~~)~~ are though sensitive requires very costly infrastructure and technical expertise (2 and 4). --

Please replace the paragraph beginning on page 2, line 5 and ending at line 17, with the following rewritten paragraph:

-- According to this invention there is provided a diagnostic kit for detecting pulmonary & extra pulmonary tuberculosis comprising a test card "TB Screen" coated with a hydrophobic material, antigen suspension, positive and ~~Negative~~ negative control.

In accordance ~~to~~ with this invention there is provided a method of detecting tuberculosis using the kit comprising

applying positive control, negative control & test sample each in circular motion on the test card zone (Fig. 3) coated with hydrophobic material adding said antigen suspension of lipsome (Fig. 4) in the presence of Sandun black 8 dye as an indicator and/or a marker to each of the positive,

negative & test sample to ~~interpart~~ interpret the results, clumping of specific antigen and anti body as dark blue agglutination was observed in positive control and the test sample which contain the active tuberculosis infection. --

Please replace the paragraphs beginning on page 2, line 19 and ending on page 3 line 21, with the following rewritten paragraphs:

-- The ~~Mycobacterium tuberculosis~~ *Mycobacterium tuberculosis* $H_{37}Rv$ (ATCC-27294) strains was grown on Sautons media till late log phase (2-3 month) and/or Middle Brook 7H9 or 7H12B both supplemented with 10% albumin dextrose and catalyst (ADC) at 37° C (2-3 weeks). The cells were harvested by centrifugation (5000,-10,000 g for 10-20 min) at 4^{o}-10^{o} C, the pellet was washed with ~~PBS (~~phosphatic buffet saline (PBS: 100mM, pH 7.2-7.6), resuspended with TEN buffer, ~~p~~PH 8.0-8.5 (10 mM Trs HC1, 1 mM EDTA, 100 mM NaCl) and heat inactivated at 70^{o}-80^{o}C (water bath) for 30-45 min. followed by sonication (15% pulse 150W) and lyphilized. The glycolipid antigens were extracted according to the procedure mentioned in the literature ~~(Reggiardo et al., 1974; Bisen P. S. et al., 2003)~~(12 and 13) with the slightly modification in the procedure. ~~The lypolized Mycobacterial~~ In brief, the sonicated and lyophilized powder of mycobacterial cells (10-15 g was taken into a glass reagent bottle and to it 100-150 ml of chloroform and methanol mixture (2:1) was added. This was stirred at room temperature for 50-60 ~~min.~~minutes and filtered through ~~whatman~~ Whatman filter paper No 1. ~~The~~ A 1/5 volume of 0.7% KCl (20.0 ml) was added to the filtrate and was uniformly shaken for 5-6 times. The suspension was transferred to a separating funnel and kept at 2-8° C. for overnight ~~till~~ until two distinct layers were separated. The lower organic phase was washed with 1/6 volume of washing solvent ~~(C:M:W:3:48:47)~~(Chloroform, methanol, and water at a ratio of 3:48:47 respectively) in similar manner by keeping at 2-8° C. for overnight. The upper aqueous phase was removed and lower organic phase was retained after filtering. The organic phase was dried by evaporating the solvent in rotatory solvent evaporator at 40-50° C. The moisture was removed by flushing the dried mixture with nitrogen gas. Neutral lipids were removed from the dried mixture by adding 300-600 ml of chilled acetone vortexing it for 10-20 ~~min~~ minutes and filtering it through ~~whatman~~ Whatman No. 1. This step was repeated ~~till~~ until the lipids in the flask became whitish or colorless. This was filtered through ~~whatman~~ Whatman No. 1 and the filtrate was discarded.

The lipids present on the filter paper were dissolved with ~~C:M~~ chloroform:methanol (2:1) and transferred to the ~~R.B~~round bottom (RB) flask. The solvent ~~Solvent~~ was rotary evaporated under reduced pressure at 40-50° C. The crude preparation was reconstituted in 10-16 ml of C:M (2:1) and stored at -20° C. for further use. --

Please replace the paragraphs beginning on page 3, line 23 and ending on page 4, line 14, with the following rewritten paragraphs:

-- The ~~Silica~~ silica gel H (S.D. Fine Chemical, India) was activated at 100-110° C. for 1-1.30 ~~hrs.~~hours (~~Hot~~ hot air oven) was packed with, glass column (2.6.times.30 cm) with manual tapping ~~and~~ in which one end was plugged with a cork and a known quantity of crude material (~~1~~ g1.0 g/5 ml, stock) was loaded on ~~either side of the column~~another side. The column was run in an ascending direction in a on chromatographic jar (4.5.times.25 cm) with 150-200 ml of purification solvent, ~~160-200 ml~~ (mobile phase) in a ratio of 66:25:4 ~~(C:M:W).sup.7,8~~ chloroform:methanol:water at room temperature ~~to run the column till other it reached the end~~following the procedure in reference 7.

The column was removed from the chromatographic jar and placed on fume hood to evaporate the solvent from the column. ~~The 1~~ A 10 cm length of each fraction was carefully scrapped using clean glass rod ~~so as to get the~~ separate the individual molecules ~~which~~ that were adsorbed with the silica gel depending upon the mobility and Retardation Factor (RF) value (46.6, 63.4, 68.3, 67.2 and 72.4%) of the individual ~~molecule~~molecules. The individual ~~fraction~~ fractions were~~was~~ collected and placed into clean dry glass test tubes, which were labeled with respective fraction number, Ten ml of extraction solvent (mixture of chloroform: ~~Methanol~~ methanol 2:1) was added to each test tubes and kept at room temperature for 30-40 ~~min.~~minutes The purity of eluted material was analyzed by TLC and the selected fraction were further filtered through Whatman filter paper No. 1 to remove the silica gel from the samples. The pure fractions were pooled and these were characterized by ~~conventional methods (Immuno-staining on TLC, ELISA and by Liposome).~~Eastern blot hybridization (Fig 1) and ELISA followed by biochemical ~~(Work and Work, 1976)~~ and immunological characterization ~~(Hives et al. 1993: Papa et al. 1989, 1993, Payne et al. 1982, Ridell et al. 1992)~~ of glycolipid antigens fractions. --

Please replace the paragraph beginning on page 4, line 15 and ending at line 30, with the following rewritten paragraph:

-- Liposome was prepared (Fig. 4) as described previously ~~(Bangham A.D. et al. 1966)~~ with ~~minor~~ some modification ~~in the procedure, .~~ ~~in~~ In brief Phophotidylcholine 100-150mg; cholesterol, 450-500 mg (Sigma, USA); antigenic suspension (Cocktail) ~~10-20~~16-25 mg; and dye 50-100 µl (1.0 -2.0 %. ~~sudan~~ Sudan black B in ~~C~~chloroform) were taken uniformly in a ~~pre-dried~~ round bottom flask while addition of 10-25ml of absolute alcohol (99.9%) Hyman, Germany). Solvent was evaporated by rotatory vacuum evaporator under reduced pressure, and. ~~The~~ the dried ~~contents were~~ mixture was further dissolved in 40-50 ml of absolute alcohol (99.9% Hyman, Germany) and were kept at 4°10°C for 1-1.30 hrs. Sucrose solution (4-8 ml; 150 Mm) was taken in a polypropylene centrifuge tube (capacity 35 mil) and to that 4-5 ml of ~~pre-prepared~~ the above alcoholic antigen suspension was gently added while vortexing. The centrifuge tubes containing the above suspension were kept over night at 4° ~~C~~-10°C for liposome swelling, vortexed with 10-15 ml of PBS (pH 6.5) buffer and centrifuged at 10, 000 g for 10-20 min (Beckman, USA). The supernatant was discarded and the pellet was resuspended with ~~20-30~~ 25-50ml of ~~B2~~ RP buffer, pH 7.2 ($NaH_2PO_4.2H_20$, 10mM; KH_2PO_4, 10mM; EDTA, 10mM; ~~Choline~~ choline ~~Chloride~~chloride, 10% and ~~Thiomersol~~thiomersol, 0.1%). This was stored at 4° ~~C~~-10°C for further use and utilized as liposomal antigen reagent for the kit. --

Please replace the paragraph beginning on page 5, line 2 and ending at line 17, with the following rewritten paragraph:

Phosphatidylcholine (PC) was prepared in house to reduce the cost of the test. Those skilled in the art are aware of purification of PC from egg yolk ~~and it's final estimation (Sunamoto, J. et al 1978)~~. In brief, 24 eggs ere taken and albumin portions were removed. The collected yolk was extracted with 750 ml of ~~Chloroform~~chloroform: ~~Methanol~~ methanol (2:1) by stirring for ~~half an hour. F~~30 minutes, and filtered through Whatman ~~no~~No. 1. The filterate was ~~deproteinised~~ deproteinized with one fifth volume of 0.7% of KC1. To the organic layer so obtained, washing was performed with 3:48:47 of ~~Chloroform~~chloroform: methanol: water. Moisture was removed by using benzene. Solvent was evaporated with the aid of rotary vacuum ~~evaporated with the aid~~

~~of rotary vacuum evaporator~~ and a dried film of lipid was obtained. Neutral lipids were removed as described above with chilled acetone. Empty weight of round bottom flask was taken ~~(Wa)~~. ~~Flask~~ The flask was weighed along with the dried lipid film. 37.5 g of crude lipid was isolated. The crude product was further purified by silica gel H chromatography and purified PC was characterized by ~~Thin~~ thin layer chromatography ~~and PC estimation was performed as known in the prior art (Sunamoto, J. et al. 1978)~~(19).

Please replace the paragraphs beginning on page 5, line 19 and continuing on page 6, line 11, with the following rewritten paragraphs:

-- ~~1 mg of Mycobacterium tuberculosis pellet was taken after centrifugation of mycobacterium tuberculosis pellet was taken after centrifugation of mycobacterial growth in Sauton's medium. The pellet was washed twice wth 1X PBS to get rid of media remnants. The pellet was then suspended in 4 ml of 1X Phosphate buffered saline. 4-8 acid washed beads of 5 mm diameter was added to the above. The sample was vigorously shaken for 10 mm on a vortex. The suspension obtained was mixed with an equal volume of Freunds Incomplete Adjuvant. The mixture was squeezed through 22g needle repeatedly till it reaches a desired level. 100 ul of suspension was inoculated to a young rabbit of 2-8 months.~~ The mixture of purified glycolipid antigens (1-2mg) in PBS (pH7.2) were emulsified with an equal volume (1.0-2.0ml) of Freunds Incomplete Adjuvant (IFA) and immunized to (2-8 months old) young rabbit subcutaneously (100-500µl/rabbit) and boosted in similar manner after 15 days interval thrice and titer was monitored (1:60-1:120) periodically. A number of rabbits were inoculated in the same manner. --

-- Rabbits were bled ~~after~~ one month after the third booster and serum was obtained. The reactivity of serum was checked with the liposome antigen suspension as described in the test procedure given in next paragraph. ~~T~~, and the reactivity titer was checked. A booster administration of the antigen (~~50~~ 100-500 µl) was again repeated. An enhanced titer of about 1:64 to 1:128 was obtained after ~~about~~ seven days to fifteen days of booster dosage. Best reactivity titer was obtained. The serum was diluted to optimum reactivity titer in 1X PBS and

0.1% sodium azide was added as a preservative, ~~so as to contain any contamination. The~~ and the stock was frozen ~~till used/dispensed in vials~~until further use. --

-- The 4-6 month old ~~Rabbits~~ rabbits were immunized with the above antigens and bled periodically. ~~and~~ The serum was oslated and used ~~for~~ as a positive control ~~to be provided with~~ for the kits, where as normal young ~~Rabbit~~ rabbit were used for ~~Negative~~ negative control. --

Please replace the paragraphs beginning on page 6, line 13 and continuing to page 7, line 4, with the following rewritten paragraphs:

-- All the components of the (TB Screen test) kit, such as positive control, negative control, liposomal antigen suspension (Fig. 2) and sample to be tested were brought to room temperature before performing the ~~experiments~~testing. ~~Positive~~ The positive control, negative control and freshly procured or frozen test serum sample (25 µl) were added and spaced evenly inside the ~~in~~ circular zone of hydrophobic material coated plastic slide (Fig. 3).~~motion, as demonstrated on the test card. The above samples were spread by using separate sticks in round conjugation.~~ Thereafter, 25 µl of liposome antigen was added to ~~it~~ each zone and the card was gently swirled for 4 ~~min~~minutes. --

-- Freshly procured or frozen test serum samples (25 µl) were spread evenly inside the circular zone of hydrophobic material coated plastic slide. For convenience, ~~zone~~ zones 1 and 2 were spread with the positive (anti-rabbit serum) and negative control (normal rabbit serum) ~~and negative control (normal rabbit serum)~~ respectively, to interpret the results. The liposome antigenic suspension (25 µl) as prepared previously was added to each circular zone including ~~zone~~ zones 1 and 2 and the card was ~~manually~~ gently swirled for 4 ~~min~~minutes. The clumping of specific antigen and antibody as dark blue agglutination were observed in positive control as well as in those samples which contain antibodies against mycobacterial glycolipid with active tuberculosis (Fig. 3A) infection.~~, No clumping on the card whereas, indicated a negative result~~and considered to be positive, where as absence of clumping on the test card were considered as negative result. The peripheral drying on the circular zone indicated ~~indiscriminate~~ indetriminate results (Fig. 3B1), which require further confirmation within 15-30

days, as these samples contained undetectable ~~level~~ levels of antigen concentration in the specimens. --

Please replace the paragraphs beginning on page 7, line 7, with the following rewritten paragraphs:

-- ~~Patient sera~~ Sera collected from outdoor patient departments (OPD) from different hospitals and pathology centers ~~of~~ in India were enrolled in the present study to cover maximum population diversity. The patients were diagnosed with TB on the basis of clinical and radiological evaluation as well as smear staining and sputum culture of samples. ~~None of the patients was complertely treated. Both extrapulmonary as well as~~ Sera samples from both categories, which include pulmonary and extra-pulmonary tuberculosis ~~sera~~ were included in the study. A total of three hundred and twenty four (324) tuberculosis sera were studied.

Sera from healthy individuals without any clinical symptoms of TB were included as negative controls to evaluate specificity criterion of the test. Most of these were obtained form BCG vaccinated subjects. The non-TB sera generally belonged either to health individuals or to patients suffering from a variety of diseases other than tuberculosis. ~~The sera~~ that were stored frozen and were used within ~~1~~ one year ~~from the time, they were taken~~. Also five hundred and eleven (511) tuberculosis negative sera were included in this testing. The details of criteria used in selection of sera is a follows:

- Smear Negative, Culture Positive pulmonary cases ---52
- Smear Positive, Culture Positive, pulmonary cases ---180
- ~~Extrapulmonary~~Extra-pulmonary, Culture Positive cases ---35
- Relapse pulmonary cases ---57
- Drug treated, clinically negative cases ---60
- Healthy household contacts ---50 --

Please replace the heading and paragraphs beginning on page 8, line 6 with the following rewritten heading and paragraphs:

-- IN HOUSE EVALUATIONS –

An overall sensitivity of 98.68% was obtained using a panoply of three hundred and twenty four tuberculosis sera, out of which 20 sera showed ~~indiscriminate~~ indeterminate results. ~~Indiscriminate sera~~ that were not included in the sensitivity and specificity calculations, as per method adopted by WHO. An overall specificity of 98.78% was obtained using five hundred and eleven non-tuberculosis sera.

Sera from 15 children who were recently immunized with BCG were tested for any cross-reactivity of the test with vaccination. None of the sera yielded positive results, thereby indicating the suitability of the test in BCG vaccinated populations ~~such as India and others~~.

~~15~~ Fifteen cases of Hepatitis B positive samples were evaluated for cross reactivity. There wqas no reaction in any of the sera tested. ~~4~~ Four Hepatitis B sera were tested with the kit at Hopkins Research Institute, ~~Bombay~~ Mumbai with nil reactivity (not included in inhouse study table).

Out of 27 sera from other common infections, 25 showed clear negative and ~~rest~~ only two showed ~~indiscriminate~~ indeterminate results. ~~There was a need to chase these~~ These subjects ~~for progression to~~ were needed to be evaluated after 15-20 days to understand the progress of tuberculosis, but unfortunately, it could not be done. Indeterminate results were omitted from specificity and sensitivity calculations. --

Please replace the sentence at the bottom of page 9 beginning with (**), with the following rewritten sentence:

-- **indiscriminate samples were not included in ~~Sensitivity~~ sensitivity & specificity calculations as per WHO methodology. --

Please replace the sentence at the bottom of page 10 beginning with (**), with the following rewritten sentence:

-- **indiscriminate samples were not included in ~~Sensitivity~~ sensitivity & specificity calculations as per WHO methodology. --

Please replace the paragraph on page 11 with the following rewritten paragraph:

-- Excellent results were obtained when using fresh sera from subjects under investigation of test results. Frozen sera can be tested after thawing, but repeated freeze thawing (more than 4-5 times) of samples might affect the outcome. --

Please add the following paragraphs after the last paragraph on page 11:

-- **References**

1. Bangham, A.D., Standish M.M, Watkins "JC Diffusion of Univalent Ions Across the Lameliae of Swollen Phospholipids, J Mol. Biol., (1965), Vol. 13, pp. 238-252.

2. Bisen, P.S., Garg, S.K., Tiwan R.P., Tagore, P.R.H., Chandra, R., Karnik, R., Thaker, N., Desai, N., Ghosh, P.K., Fraziano, M., Colizzi, V.; "Analysis of Shotgun Expression Library of Mycobacterium Tuberculosis Genome for Immunodominant Polypeptide: Potential Use in Serodiagnosis", Clin and Diagn. Lab Immunol., (2003), Vol. 6, pp 1051-1058.

3. Charpin, D.H., Gevaudan, H., Saadjian, M.J., De Micco, P., Arnaud, A., Vervloet, D., Charpin, J., "Value of ELISA Using A-60 Antigen in the Diagnosis of Active Pulmonary Tuberculosis", Am. Rev. Respir. Dis., (1990), Vol. 142, pp. 380-388.

4. Garg, S.K., Tiwan, R.P., Tiwan, D., Singh, R., Malhotra, D., Ramnani, V.K., Prasad, G.B.K.S., Chandra, R., Fraziano, M., Colizzi, V., Bisen, P.S., "Diagnosis of Tuberculosis Available Technologies, Limitations and Possibilities", J. Clin. Lab Anal., (2003), Vol. 17, pp. 155-163.

5. Hines, M.E., Jaynes, J.M., Barker, S.A., Newton, J.C., Enright, F.M., Snider, T.G., "Isolation and Partial Characterization of Glycolipid Fractions from *Mycobacterium avium* serovar 2 (*Mycobacterium paratuberculosis* 18) that Inhibit Activated Macrophages Infection and Immunity", (1993) Vol. 61, pp. 1-7.

6. Konstantin, P.K., Claudia, L.M., Roberto, C., Anna, H., Alan, W., Gennaro, M.L., "Use of *M. tuberculosis* Complex-specific Antigen Cocktails for a Skin Test Specific for Tuberculosis Infection and Immunity", (1998), Vol. 66, pp. 3606-3610.

7. Lepage, M., "Isolation and Characterization of an etherified form of Sterile Glucoside", J. Lipid Res., (1964), Vol. 53, pp. 587-592.

8. Papa, F., Cruaud, P., Luquin, M., Torei, M.F., Gohand, K.S., David, H.L., "Isolation and Characterization of Serologically Reactive Lipooligosaccharides from *M. tuberculosis*", Res. Microbiol., (1993), Vol. 144, pp. 91-99.

9. Papa, F.P., Cruaud, P., David, H.L., "Anigenicity and Specificity of Selected Glycolipid Fractions from *M. tuberculosis*", Res. Microbiol., (1989), Vol. 140, pp. 569-578.

10. Payne, S.N., Draper, P., Rees, R.J.W., "Serological Activity of Purified Glycolipid from *Mycobacterium leprae"*, Int. J. Lepr., (1982), Vol. 50, pp. 220-221.

11. Ridell, M., Wallestrom, G., Minhikin, D.E., Bolton, R.C., Magnusson, M.A., "Comparative Serological Study of Antigenic Glycolipids from *M. tuberculosis"*, Tubercle Lung Dis. (1992), Vol. 73, pp. 101-105.

12. Reggiardo, Z., Middlebrook, G., "Serologically active glycolipid Families from Mycobacterium Bovis BCG I. Extraction, purification and immunologic studies", Am. J. Epidemiol., (1974a), Vol., 100, pp. 469-476.

13. Reggiardo, Z., Middlebrook, G., "Serologically active glycolipid Families from Mycobacterium Bovis BCG II. Serologic studies on human sera", Am. J. Epidemiol., (1974b), Vol., 100, pp. 477-486.

14. Somi, G.R., Grien, R.J.O., Mfinanga, G.S., Ipuge, Y.A., "Evaluation of the Mycodor™ Test in Patients with Suspected Tuberculosis in a Field Setting in Tanzania", Int. J. Tubercle and Lung Disease, (1999), Vol. 3, pp. 231-238.

15. Work, T.S., Work, E., "Handbook of Laboratory Techniques in Biochemistry and Molecular Biology", Published by North-Holland Publishing Co., Inc., New York, (1975), Vol. 3.